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# Competition Model for Upregulation of the Major Histocompatibility Complex Class II-Associated Invariant Chain by Human Immunodeficiency Virus Type 1 Nef[▽]

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**The human immunodeficiency virus type 1 (HIV-1) Nef protein upregulates the expression of the invariant chain (Ii)/major histocompatibility complex class II (MHC-II) complex at the cell surface. This complex appears to reach the antigen-loading endosomal compartment at least in part via an indirect pathway in which it is internalized from the cell surface via the adaptor protein 2 (AP-2) complex. Here we provide evidence for a competition model to explain how Nef upregulates the expression of Ii at the cell surface. In this model, Nef and Ii compete for binding to AP-2. In support of this model, Nef decreased the rate of internalization of Ii from the cell surface. The AP-binding dileucine motif in Nef, $ENTSLL_{165}$, was necessary and sufficient for the upregulation of Ii. In addition, two leucine-based AP-binding motifs in the Ii cytoplasmic tail, $DDQRDLI_8$ and $EQLPML_{17}$, were critical for the efficient upregulation of Ii by Nef. Experiments using Nef variants in which the native dileucine-based sorting motif was replaced with similar motifs from cellular transmembrane proteins allowed modulation of AP-binding specificity. Analysis of these variants suggested that the binding of Nef to AP-2 is sufficient to upregulate Ii at the plasma membrane. Finally, interference with the expression of AP-2 caused an upregulation of Ii at the plasma membrane, and this decreased the effect of Nef. These data indicate that Nef usurps AP-2 complexes to dysregulate Ii trafficking and potentially interfere with antigen presentation in the context of MHC-II.**

The human immunodeficiency virus type 1 (HIV-1) Nef protein is critical for efficient pathogenesis and progression to AIDS (25). How Nef causes this is unclear, but missorting of cellular membrane proteins may play a key role by contributing to optimal virion infectivity and by enabling viral evasion of adaptive immunity (40). Specifically, Nef downregulates the expression of CD4 and major histocompatibility complex class I (MHC-I) at the cell surface. Nef also downregulates mature MHC-II and upregulates immature MHC-II at the cell surface (47). The MHC-II complex is formed in the endoplasmic reticulum by the noncovalent association of the α and β subunits to form a binding pocket (45). Initially, this pocket is occupied by the invariant chain protein (Ii), and the complex is referred to as immature MHC-II. Ii is a type II transmembrane protein that acts as a chaperone, directing the complex to a late endosomal/lysosomal compartment (referred to as the MHC-II compartment or MIIC) in which Ii is proteolyzed and an antigenic peptide takes its place in the peptide-binding pocket. This complex, now referred to as mature MHC-II, traffics to the plasma membrane (PM), where it presents the antigenic peptide to the T-cell receptor of a $CD4^+$ T cell (19). Evidence exists for two pathways that the immature MHC-II complex may travel en route to the antigenic peptide-loading compartment. The direct pathway is from the trans-Golgi network (TGN) to a late endosome (1, 9). The indirect pathway involves transport from the TGN to the PM, followed by rapid internalization and transport to a late endosomal compartment (17, 39).

Leucine-based sorting signals have been found in the cytoplasmic tails of a number of eukaryotic membrane proteins, including Ii and Nef (7, 38). These signals bind to adaptor protein (AP) complexes (18, 26). AP complexes link the membrane protein to clathrin, which, along with accessory proteins, coats the cytoplasmic face of transport vesicles. The AP complex family has four members. The AP-1 complex is involved in the transport of vesicles from the TGN to the endosome, while the AP-2 complex is specific for vesicles forming at the PM. The AP-3 complex is mainly associated with endosomal membranes and the AP-4 complex associates with the TGN (26). AP complexes are composed of four subunits, a large chain (~100 kDa) specific to the type of AP complex (γ for AP-1, α for AP-2, δ for AP-3, and ε for AP-4), and three subunits with significant homology, a large chain (β1, β2, β3, and β4 [~100 kDa]), a medium chain (μ1, μ2, μ3, and μ4 [~50 kDa]), and a small chain (σ1, σ2, σ3, and σ4 [~25 kDa]).

The binding of Ii to AP complexes and the roles of these interactions have been investigated. The cytoplasmic tail of Ii bound to AP-1 and AP-2 but not to AP-3 as measured by surface plasmon resonance (21). The binding of Ii to AP-1 and AP-2 was dependent on the leucine signals, $DDQRDLI_8$ and

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$EQLPML_{17}$. If only one of the leucine-based signals was mutated (either $L_7A$ or $L_{17}A$), binding with high affinity to AP-1 or AP-2 still occurred; if both signals were mutated ($L_7AL_{17}A$), no binding was found between Ii and AP-1 or AP-2 (27). When Ii/MHC-II trafficking was examined in *mocha* mice, which do not express functional AP-3, no differences were found compared to mice that do express AP-3 (43). Likewise, in human B-lymphoblast lines deficient in the AP-3 complex, normal trafficking of MHC-II was observed (3). These in vivo data are consistent with the lack of binding of Ii to AP-3 in vitro. Recent experiments using RNA interference (RNAi) suggest that AP-2 may be the key AP complex that controls the trafficking of Ii (11, 33). Knockdowns of AP-1 had little to no effect on immature MHC-II trafficking, yet depletion of AP-2 (as well as clathrin) resulted in increased expression of the immature MHC-II complex at the cell surface.

Nef has been shown to interfere with antigen presentation in the context of MHC-II and to increase the surface expression of the Ii/MHC-II complex (47). This effect has been seen with Nef proteins of HIV-1, HIV-2, and simian immunodeficiency virus (42) and when Nef proteins were expressed in various cell lines, including HeLa-CIITA and Mel JuSo, as well as in primary cultures of human cells, including $CD4^+$ T cells (24, 41, 42) and monocyte-derived macrophages (41, 42). The upregulation of Ii (as well as the downregulation of CD4) requires two leucine residues within a C-terminal flexible loop of the Nef protein (47), which are also necessary for binding to AP complexes (5). Although models for the downregulation of cell surface proteins have been proposed in which Nef links the cytoplasmic tail of CD4 to AP-2 (4) and that of MHC-I to AP-1 (49), a model for the upregulation of immature MHC-II and the roles of the specific AP complexes are undefined. In the present study, we present evidence in support of a model in which Nef competes with Ii for AP-2, resulting in upregulation of Ii at the cell surface.

## MATERIALS AND METHODS

**Cells and transfections.** Peripheral blood mononuclear cells were isolated from blood obtained from healthy donors by centrifugation through a Histopaque-1077 gradient (Sigma-Aldrich, St. Louis, MO). $CD4^+$ T cells were isolated from peripheral blood mononuclear cells with the CD4 T-cell isolation kit RosetteSep (StemCell Technologies, Vancouver, Canada) as recommended by the manufacturer. HeLa clone P4.R5 cells were a gift from Ned Landau and were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) and supplemental glutamine, penicillin, streptomycin, and puromycin (1 μg/ml). HeLa-CIITA cells were a gift from Philippe Bénaroch and were maintained in Dulbecco's modified Eagle's medium with 10% FBS and supplemental glutamine, penicillin, streptomycin, nonessential amino acids, and hygromycin B (300 μg/ml). HeLa-CIITA cells are HeLa cells stably transfected with the gene for the MHC-II transcriptional activator, allowing the expression of genes required for MHC-II presentation, e.g., HLA-DR, Ii, and HLA-DM (47). HeLaP4.R5 and HeLa-CIITA cells were transfected with cationic lipid Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

**Infections.** Cell-free virions were produced by transfection of HeLa-P4.R5 cells in a 10-cm dish with 24 μg of the indicated proviral plasmid (pNL4-3 or pDS, NL4-3 with two stop codons in the *nef* gene). Forty-eight hours later, cell-free supernatants were harvested and stored at −80°C. On the day of infection, the frozen virions were thawed and concentrated by ultracentrifugation in a Beckman Avanti 30 tabletop ultracentrifuge (23,000 × *g*, 2 h, 4°C). The viral pellet was resuspended in Roswell Park Memorial Institute medium (RPMI)–10% FBS. Two microliters of anti-CD44 MicroBeads (Miltenyi Biotec GmbH, Gladbach, Germany) was added per 100 μl of virions, and the mixture was incubated at room temperature for 1 h. Primary human $CD4^+$ T cells were pelleted by centrifugation at ~300 × *g* for 10 min. Cell pellets were resuspended in the concentrated virions (plus anti-CD44 MicroBeads [~3 million cells/ml], WT NL4-3 [~23 μg pelletable p24 per 1.5 million cells], and ΔNef NL4-3 [~31 μg pelletable p24 per 750,000 cells]) and incubated overnight at 37°C and 5% $CO_2$. The next day, the pelleted cells were resuspended in RPMI–10% FBS–3 μg/μl phytohemagglutinin and incubated overnight as before. The following day, the medium was replaced with RPMI–10% FBS–20 U/ml interleukin-2 (IL-2). Three days later, the cells were harvested, stained for surface expression of Ii, and analyzed by flow cytometry.

**Abs.** The antibodies (Abs) used were phycoerythrin (PE)-conjugated murine anti-CD74 (Ii) clone M-B741 (Ancell Corp., Bayport, MN), murine anti-CD74 clone M-B741 (Becton Dickinson, San Jose, CA), fluorescein isothiocyanate (FITC)-conjugated murine anti-CD25 (Tac antigen/IL-2 receptor α chain; BD Biosciences, San Jose, CA), FITC-conjugated murine anti-HIV-1 p24 (Beckman Coulter, Fullerton, CA), a goat anti-mouse secondary Ab conjugated to allophycocyanin (APC; Jackson ImmunoResearch, West Grove, PA), and anti-γ, anti-μ2, and anti-δ Abs (BD Transduction Laboratories, San Jose, CA).

**Plasmids.** The HIV-1 NL4-3 proviral *nef* gene was PCR subcloned into the expression vector pCIneo (Promega, Madison, WI) and referred to as pCINL (7). Mutations were introduced previously into the ENTSLL sequence of pCINL (5, 6). Green fluorescent protein (GFP) was expressed from pCG-GFP (15). pCDM8-TAC vectors were prepared as described previously. (7, 32). The pRSV5-Ii vector was a gift from Philippe Bénaroch. Mutations were made in the Ii gene by overlap PCR (20).

**Flow cytometry.** Approximately 24 h after transfection, HeLaP4.R5 or HeLa-CIITA cells were removed from plates with 0.5 mM EDTA–1× phosphate-buffered saline (PBS) and stained for 1 h at 4°C with a PE-conjugated anti-CD74 (Ii) monoclonal Ab (MAb), an FITC-conjugated anti-CD25 MAb, or an unlabeled anti-CD74 MAb. The cells were washed in 1× PBS–0.1% azide–2% FBS and fixed in 1% paraformaldehyde, while cells stained with the unlabeled Ab were stained with an APC-conjugated secondary Ab for 60 min at 4°C, washed, and fixed in 1% paraformaldehyde. For combined analysis of surface and intracellular HIV-1 p24 in primary $CD4^+$ lymphocytes, unfixed cells were stained first for surface Ii with PE-conjugated anti-CD74 MAb and then fixed and permeabilized with the Cytofix/Cytoperm kit (BD Biosciences), followed by staining with FITC-conjugated anti-p24 MAb. Analysis of total Ii expression in HeLa-CIITA cells was performed with the Cytofix/Cytoperm kit and staining with a PE-conjugated anti-CD74 MAb. A gate on a forward-scatter and side-scatter plot allowed for exclusion of dead cells in the analysis.

**RNAi.** HeLa-CIITA cells were initially transfected with small interfering RNA (siRNA) duplexes (Dharmacon, Lafayette, CO) by using Lipofectamine 2000. Three days later, the cells were cotransfected with siRNA and plasmid DNA ([i] pCIneo or pCINL and [ii] pCG-GFP). The cells were processed for flow cytometry 3 days after the second round of transfections. The following sequences were used to generate RNAs specifically targeting human AP mRNAs and were ordered from Dharmacon as 21-nucleotide duplexes with 3′ dTdT overhangs as follows: μ1, 5′-AAGGCAUCAAGUAUCGGAAGA-3′; μ2, 5′-AAGUGGAUGCCUUUCGGGUCA-3′; μ3, 5′-AAGGAGAACAGUUCUUGCGGC-3′. For control experiments, we used siCONTROL nontargeting siRNA 1 (Dharmacon). Immunoblot assays were performed as follows. Cells treated with siRNA directed against μ1, μ2, or μ3 or with a nontargeting siRNA were lysed in a buffer containing 5% Triton X-100 and protease inhibitors. Nuclei and cellular debris were cleared from the lysate by centrifugation. Lysates were run on a 12% polyacrylamide gel (Bio-Rad, Hercules, CA), blotted onto a polyvinylidene difluoride membrane, and blocked overnight in 4% nonfat dry milk. The membrane was probed against AP-1 γ, AP-2 μ2, and AP-3 δ subunits separately. The blots were stripped overnight and reprobed for tubulin.

**Internalization assay.** HeLa-CIITA cells were transfected with a plasmid expressing Nef or the parental plasmid, pCIneo, and pCG-GFP. Approximately 24 h later, cells were detached with 0.5 mM EDTA–PBS, stained with an anti-CD74 Ab for 1 h at 4°C, and washed with 2% FBS–PBS. Cells were separated into six aliquots, with one aliquot, designated $t = 0$, stained with an APC-conjugated secondary Ab at 4°C. The remaining five aliquots were incubated at 37°C and 5% $CO_2$, and at various times, ice-cold 1× PBS–0.1% azide–2% FBS was added and the cells were placed on ice and stained with an APC-conjugated secondary Ab. Cells were washed and fixed in 1% paraformaldehyde and analyzed by flow cytometry.

**Yeast transformation, Y3H assays, and growth curves.** Yeast three-hybrid (Y3H) assays were performed as previously described (4, 6, 23). Briefly, wild-type (WT) and mutant versions of NL4-3 Nef were expressed as GAL4 binding domain fusion proteins from the pBridge vector (Clontech, Mountain View, CA) along with rat σ1A, σ2, or σ3A. Mouse γ1, rat αC, and human δ were expressed as GAL4 activation domain fusion proteins from the pGADT7 vector (Clon-

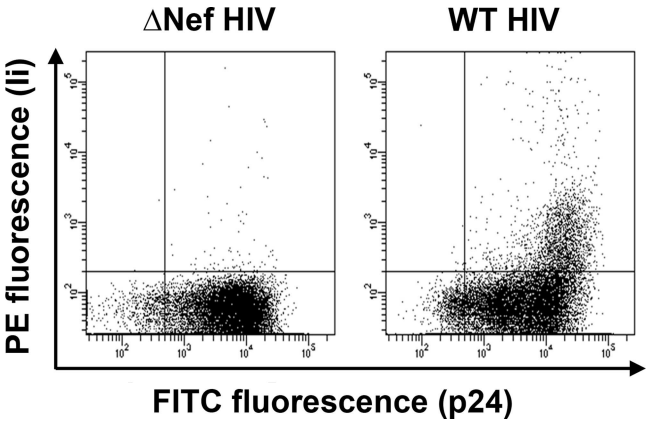


FIG. 1. Nef-mediated upregulation of cell surface Ii expression in HIV-infected primary human $CD4^+$ T cells. $CD4^+$ T cells were isolated from human blood and infected with concentrated HIV-1 NL4-3 or ΔNef HIV-1 NL4-3 that does not express Nef. Cells were exposed to the virus for ~18 h, stimulated with phytohemagglutinin for a day, and grown in the presence of IL-2 for 3 days prior to harvesting and staining for analysis by flow cytometry. Cell surfaces were stained with an anti-Ii Ab conjugated to PE, and cell cytoplasm was stained with an anti-p24 Ab conjugated to FITC.

tech). Pairs of pBridge and pGADT7 plasmids were used to transform the *Saccharomyces cerevisiae* HF7c strain by the lithium acetate procedure, and positive transformants were selected on agar plates lacking Leu, Trp, and Met. For the plate-based Y3H assays, colonies from each sample were pooled, normalized to equivalent concentrations, and transferred to three sets of plates, i.e., those lacking Leu, Trp, and Met, those lacking His, Leu, Trp, and Met, and those lacking His, Leu, Trp, and Met, and supplemented with 3 mM 3-amino-1,2,4-triazole (3AT). Growth of the transformed yeast colonies on solid medium was assayed 4 days later. For the quantitative growth curves, positive transformants were cultured overnight in liquid dropout medium lacking Leu, Trp, and Met; normalized to equivalent concentrations; and washed twice with water. The yeast cells were then resuspended in equal volumes of liquid dropout medium lacking His, Leu, Trp, and Met and cultured in triplicate at 30°C. The optical density at 600 nm of each culture was measured at regular intervals over the course of 24 h.

## RESULTS

**Nef upregulates Ii in HIV-1-infected primary human $CD4^+$ T cells.** Stumptner-Cuvelette and colleagues initially demonstrated Nef-mediated upregulation of Ii in HeLa-CIITA and MelJuSo cells (47). The former are HeLa cells stably transfected to produce CIITA, which induces the expression of the MHC-II α and β subunits, HLA-DM, and Ii. MelJuSo cells are a melanoma cell line that is known to express MHC-II and Ii (47). Nef is also known to upregulate the surface expression of Ii in primary human $CD4^+$ T cells infected with a vesicular stomatitis virus G-pseudotyped HIV (24, 41, 42). To test the relevance of Nef-mediated upregulation of Ii under more physiological conditions, we infected human primary $CD4^+$ T cells with HIV-1 NL4-3 (WT) and an HIV-1 NL4-3 mutant that does not express the *nef* gene (ΔNef) and assayed the expression of Ii at the cell surface by flow cytometry. Figure 1 shows a striking increase (~15-fold) in cell surface Ii expression in cells infected with WT NL4-3 HIV-1 compared to the ΔNef HIV-1 mutant. Thus, Nef increases the expression of Ii at the surface of HIV-1-infected human primary $CD4^+$ T cells, which are the primary target of HIV-1 in vivo.

**The dileucine (ENTSLL) motif in Nef is both necessary and sufficient for the upregulation of Ii.** Nef has a disordered loop near its carboxy terminus that contains a canonical dileucine sorting signal, D/ExxxLL (40). This sequence is ENTSLL in the Nef protein encoded by HIV-1 NL4-3. One model that can explain the ability of Nef to upregulate Ii is that it competes for the cellular sorting machinery that would normally direct immature MHC-II to the MIIC, leading to accumulation of Ii at the cell surface by default. In this model, the Nef ENTSLL sequence is not only necessary but also sufficient to upregulate Ii. To determine whether the Nef dileucine motif (ENTSLL) by itself could mediate the upregulation of Ii, this sequence was fused to the carboxy-terminal cytoplasmic domain of the transmembrane protein Tac (IL-2 receptor α chain). HeLa-CIITA cells were cotransfected with a plasmid encoding Tac or various Tac chimeras (Fig. 2). Tac chimeras containing a D/ExxxLL motif, from either the CD3γ cytoplasmic tail (DKQTLL) or Nef (ENTSLL), upregulated the expression of Ii at the cell surface by approximately eightfold in the case of Tac-DKQTLL and sixfold in the case of Tac-ENTSLL. A Tac chimera with the Nef motif mutated by replacement of the leucines with alanine did not upregulate the cell surface expression of Ii. Thus, the ENTSLL motif of Nef from HIV-1 NL4-3 is not only necessary but also sufficient for the upregulation of Ii.

**Surface expression of Ii in isolation is affected by Nef in the same manner as Ii in complex with MHC-II.** In previous work, we performed a mutational analysis of the Nef dileucine sorting signal and measured Nef-induced upregulation of Ii expression at the cell surface by flow cytometry with HeLa-CIITA cells, which stably express both subunits of MHC-II, Ii, and HLA-DM (5). To determine whether these relationships are manifest when Ii is expressed in isolation from the rest of MHC-II, HeLa cells were transfected to express Ii and Nef transiently (Fig. 3A). The extent of upregulation of Ii by Nef was slightly greater when it was expressed in isolation (approximately 13-fold, Fig. 3A) than when it was expressed with the complete MHC-II (approximately four-fold; Fig. 3B), but the requirements within the Nef-ENTSLL sequence were the same: mutation of the leucines abrogated activity, whereas mutation of $E_{160}$ decreased activity by >50%. These data suggest that the effect of Nef on the trafficking of the Ii/MHC-II complex is represented accurately when Ii alone is expressed.

**Nef upregulates total cellular Ii expression modestly, with the majority due to upregulation at the PM.** To exclude the possibility that the effect of Nef on the expression of Ii at the PM could be attributed to an increase in the total cellular accumulation of Ii at steady state, we measured total Ii expression (PM and cytoplasmic) with an intracellular-fluorescence-activated cell sorting-based assay. In this assay, the PM is permeabilized with detergent before Ab staining, so that Ii both within the cell and on the cell surface is detected. We also stained nonpermeabilized cells to measure expression at the PM exclusively. Approximately 24 h after the cotransfection of HeLa-CIITA cells with a Nef-expressing plasmid or an empty vector and a GFP-expressing plasmid, cells were processed as described in Materials and Methods for intracellular and PM fluorescence-activated cell sorter analysis. As shown in Fig. 4, Nef caused a slight increase in total Ii expression (1.5-fold) but most of this could be attributed to the increase in Ii at the PM

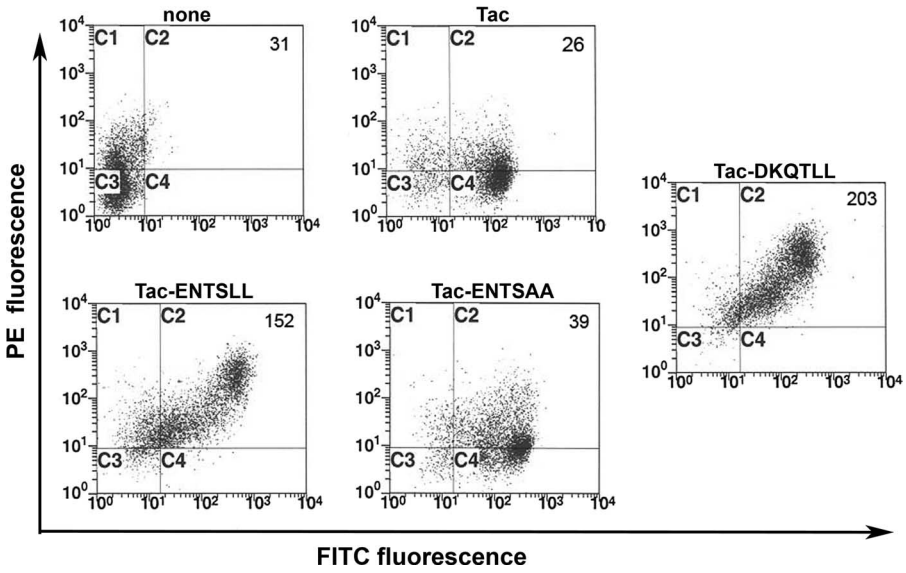


FIG. 2. Tac chimeras with dileucine motifs are able to upregulate cell surface expression of Ii. HeLa-CIITA cells were transfected with plasmids encoding the Tac chimeras. Tac is the IL-2 receptor α chain. As indicated, the sequences (DKQTLL, ENTSLL, and ENTSAA) were fused to the C terminus of the Tac cytoplasmic tail. The sample marked "none" is a control consisting of untransfected HeLa-CIITA cells. Tac was identified by an Ab conjugated to FITC, while Ii was recognized by an Ab conjugated to PE. The PE MFI of the $FITC^+$ cells is the value in the C2 quadrant.

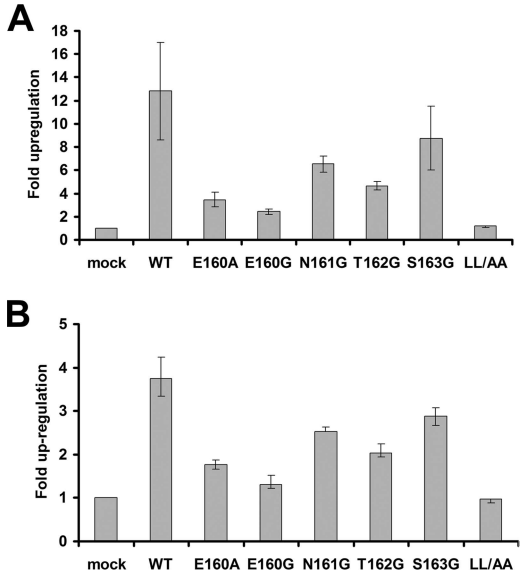


FIG. 3. Effect of dileucine motif mutant Nef proteins on cell surface Ii expression in HeLa cell lines. Mutations were made in the dileucine motif of Nef (NL4-3 $ENTSLL_{165}$). Plasmids encoding these mutant Nef proteins were transfected along with a marker plasmid expressing GFP into (A) HeLa P4.R5 (Ii) cells or (B) HeLa-CIITA (Ii and MHC-II) cells. Twenty-four hours later, the cells were stained for Ii at the cell surface with an anti-Ii Ab conjugated to PE, washed, fixed, and analyzed by flow cytometry. The PE MFI of the $GFP^+$ cells is plotted normalized to a mock treatment value set to 1. For the HeLa P4.R5 cells (A), MFI values from two independent experiments were averaged. For the HeLa-CIITA cells (B), MFI values of duplicate samples from one experiment were averaged. Error bars represent the actual data values (used to compute the average MFI).

(7-fold). Similar increases in total and surface expression of Ii in the presence of Nef have been reported by Stumptner-Cuvelette et al. (46). These data suggest that the upregulation of Ii at the cell surface by Nef is not likely a secondary effect of an increase in the total cellular levels but more likely reflects a specific effect on the trafficking of Ii.

**Nef slows the rate of Ii internalization.** The relatively low levels of expression of Ii at the cell surface are due in part to a rapid rate of internalization (2). To test the hypothesis that Nef might interfere with this, the rate of disappearance of Ii from the cell surface was measured in the presence and absence of Nef. HeLa-CIITA cells were cotransfected with plasmids expressing GFP and Nef or an empty vector. One day later, cells were stained with an anti-Ii chain Ab at 4°C. The cells were washed, incubated at 37°C for different times, stained with a secondary Ab conjugated to APC, fixed, and

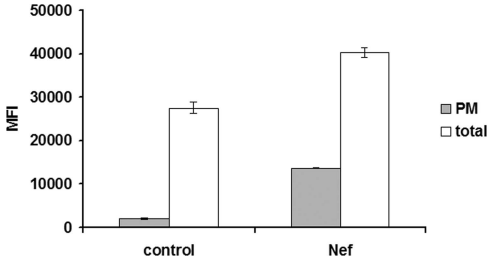


FIG. 4. Effect of Nef on surface and total cellular expression of Ii. HeLa-CIITA cells were transfected with (i) a plasmid that expresses Nef or an empty control plasmid and (ii) a plasmid expressing GFP as a transfection marker. Cells were assayed for intracytoplasmic staining (total) and surface expression of Ii as described in Materials and Methods. The average APC MFI from duplicate samples from one experiment is shown on the *y* axis, with error bars indicating the actual data values (used to compute the average MFI).

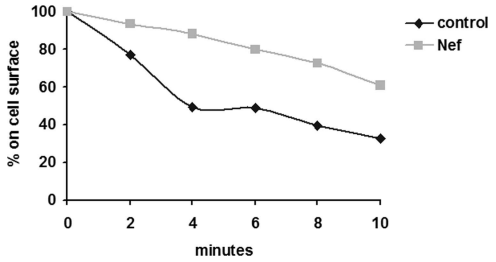


FIG. 5. Kinetics of cell surface internalization of Ii. HeLa-CIITA cells were transfected with a plasmid encoding WT Nef, or a control plasmid, and GFP. One day later, the cells were stained with a primary Ab against Ii and then placed at 37°C for various times before staining with a secondary Ab conjugated to APC. The APC MFI of $GFP^+$ cells was measured over time, with $t = 0$ set to 100%.

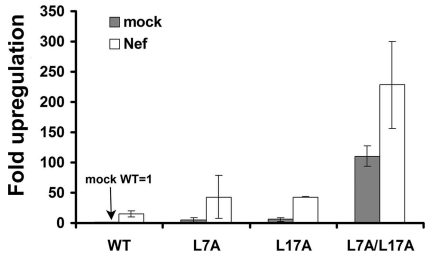


FIG. 6. Nef-mediated upregulation of mutant Ii proteins with alanine substitutions in leucine-based sorting signals. HeLa P4.R5 cells were transfected with plasmids that expressed mutated Ii, WT Nef, and GFP. Three mutant Ii chains were tested, i.e., $L_7A$ (membrane-distal sorting signal), $L_{17}A$ (membrane-proximal signal), and $L_7AL_{17}A$ (both signals mutated). Twenty-four hours after transfection, the cells were stained for cell surface Ii expression with a primary Ab, followed by a secondary Ab conjugated to APC. The *y* axis shows the average, from two independent experiments, of the APC MFI for $GFP^+$ cells normalized to WT Ii without Nef (mock WT) set to 1. Error bars indicate the actual data values (used to compute the average MFI).

analyzed by flow cytometry. The expression of Ii at the cell surface was measured after 0, 2, 4, 6, 8, and 10 min of incubation at 37°C. Nef upregulated cell surface Ii expression sixfold (data not shown). Although it did not abrogate the internalization of Ii, Nef decreased the fractional rate of internalization of Ii (Fig. 5, in which the mean fluorescence intensity [MFI] values at zero time for samples either with or without Nef were set to 100%). This effect was greatest at early time points; for example, after 4 min of incubation at 37°C, >50% of the Ii protein had been internalized in the absence of Nef but only 10% had been internalized in the presence of Nef. These data indicate that Nef decreased the fractional rate of Ii internalization.

**Optimal upregulation of Ii at the cell surface by Nef requires leucine-based sorting signals in the Ii cytoplasmic tail.** As noted above, Ii has leucine-based signals, $DDQRDLI_8$ and $EQLPML_{17}$, in its cytoplasmic tail that direct internalization from the cell surface. In a competition model in which Nef usurps the cellular machinery recognized by Ii, mutations within these signals should decrease the effect of Nef. We constructed mutations in the leucine-based sorting signals in the cytoplasmic tail of Ii (Table 1). Previous research demonstrated that the two signals function independently as internalization signals from the PM (2). If either the membrane-distal signal ($L_7A$) or the membrane-proximal signal ($L_{17}A$) is mutated, Ii is still internalized from the cell surface and directed to the endocytic pathway. However, if both signals are mutated ($L_7AL_{17}A$), Ii remains at the cell surface (2, 34, 35).

We assayed the expression of these mutant proteins on the HeLa cell surface in the presence and absence of Nef by flow cytometry. Plasmids encoding Ii, GFP, and Nef and the empty parental plasmid were cotransfected into these cells (Fig. 6). In the absence of Nef, mutation of the leucine residue at either position 7 or 17 to alanine resulted in an approximately fivefold increase in the cell surface expression of Ii. Ii with leucine-to-alanine mutations at both positions 7 and 17 ($L_7AL_{17}A$) was expressed at the cell surface at levels >100-fold higher than that of the WT. As shown previously, Nef increased the expression of Ii by approximately 15-fold (47). In contrast, Nef increased the surface expression of Ii with either the $L_7A$ or the $L_{17}A$ mutation by 7- to 10-fold and increased the surface expression of the double mutant $L_7AL_{17}A$ by only 2-fold. These data indicate that the optimal effect of Nef requires the leucine-based sorting signals in the cytoplasmic domain of Ii, although a small twofold upregulation persists even when both of these signals are disrupted.

TABLE 1. Mutant Ii constructs

| Construct | Cytoplasmic tail sequence |
|---|---|
| WT | MDDQRDLISNNEQLPMLGRRPGAPESKCSR |
| L7A | ------A---------- |
| L17A | ----------A---------- |
| L7AL17A | ------A---------A---------- |

**Mutant Nef proteins with dileucine motifs from cellular transmembrane proteins bind differentially to AP complexes and upregulate Ii with different activities.** The preceding data suggest that if a competition exists between Nef and Ii for access to the cellular sorting machinery, it likely involves leucine-based motifs in each protein. These motifs bind the heterotetrameric AP complexes (18, 26). At least some of these motifs, including that in Nef, appear to bind to hemicomplexes consisting of the large specific and small subunits of the heterotetramer (4, 6, 10, 23). To correlate the binding of Nef to specific AP complexes with its ability to upregulate Ii, we substituted leucine-based motifs from cellular proteins into Nef and then tested these mutant proteins both in an AP-binding assay and for their activity in modulating the surface expression of Ii.

We previously constructed Nef proteins in which the dileucine motif of NL4-3 Nef, ENTSLL, was replaced with the leucine-based sorting signals found in the cytoplasmic tails of cellular transmembrane proteins, specifically, GDKQTLL from CD3γ or EERQPLL from tyrosinase (6). Here, the binding between these mutant Nef proteins and the hemicomplexes of AP-1, -2, and -3 was measured with a Y3H assay (Fig. 7A). Although Nef has been reported to bind to different AP complex subunits, i.e., μ1 (7, 12, 29), μ2 (2, 7, 36), β1, β2 (14), and hemicomplexes of γ/σ1 (6, 10, 23), α/σ2 (4, 10), and δ/σ3 (6, 23), binding to the AP hemicomplexes is mediated by the ExxxLL motif and has been associated with functions that depend on this motif by mutational analysis. In the Y3H assay, growth of yeast in the absence of histidine (−His) indicates

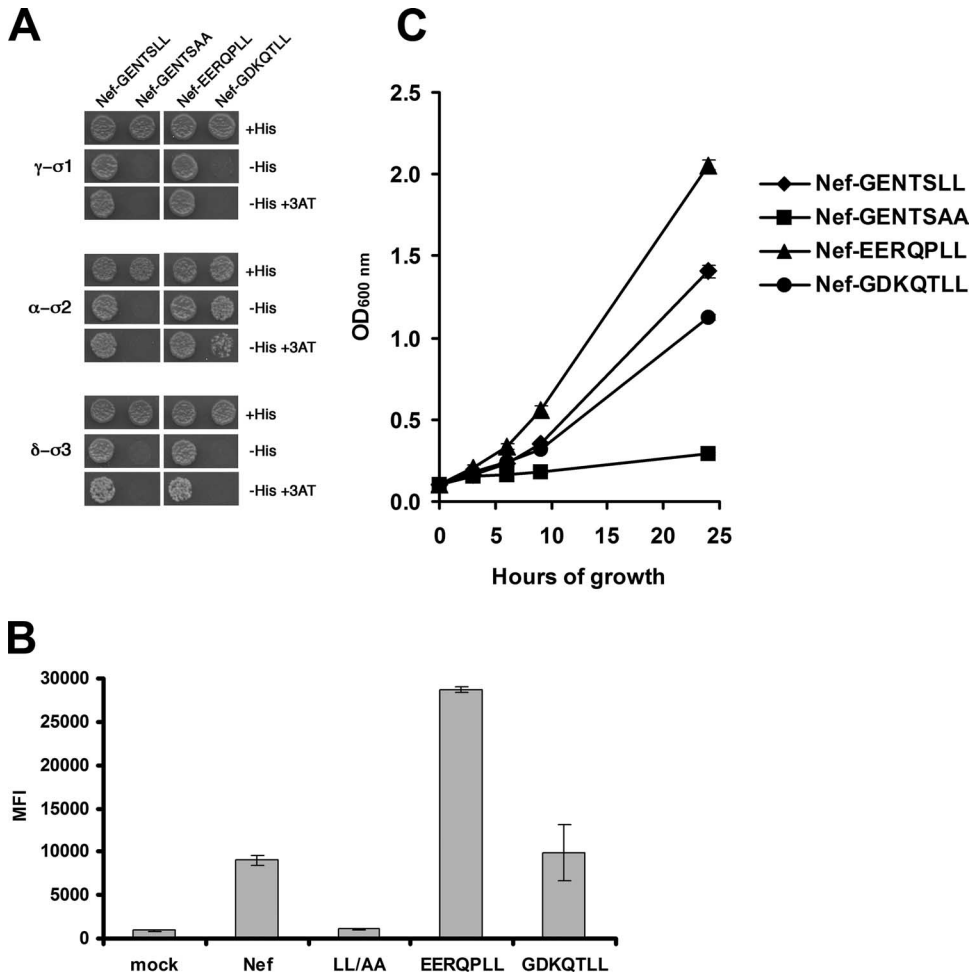


FIG. 7. Binding to hemicomplexes of AP-1, -2, and -3 and Ii upregulation by mutant Nef containing heterologous leucine-based sorting motifs. (A) In the Y3H assay shown, WT (GENTSLL) and mutant (GENTSAA, EERQPLL, and GDKQTLL) versions of Nef were fused to the GAL4 binding domain and expressed along with σ1, σ2, or σ3 from the pBridge vector, while γ, α, and δ were expressed as GAL4 activation domain fusion proteins from pGADT7. Growth of HF7c yeast on plates lacking histidine (−His) is indicative of an interaction, whereas growth on plates lacking histidine and containing 3 mM 3AT (−His +3AT) indicates interaction at a higher level of stringency. Growth on plates containing histidine (+His) is a control for yeast cotransformation and viability. The data are representative of two independent experiments. (B) Upregulation of Ii at the PM by WT and mutant Nef proteins. HeLa-CIITA cells were transfected with plasmids encoding the substitution-containing Nef proteins and a plasmid encoding GFP. Twenty-four hours later, the cells were stained for Ii with a primary Ab and then a secondary Ab conjugated to APC. The average APC MFI of $GFP^+$ cells in two independent experiments is plotted for each sample; error bars indicate the actual data values (used to compute the average MFI). Mock, empty parental plasmid; Nef, WT Nef; LL/AA, Nef with $LL_{165}$ replaced with AA; EERQPLL, Nef with the EERQPLL sequence (tyrosinase); GDKQTLL, Nef with the GDKQTLL sequence (CD3γ). (C) Quantitative analyses of the interactions between Nef or dileucine mutant Nef proteins and the AP-2 hemicomplex. Quantitative Y3H assays were conducted by measuring the growth rate of cotransformed yeast in selective liquid medium lacking histidine. Yeast cells were cultured in triplicate for each cotransformation, and the average optical density (OD) at 600 nm for each time point is plotted with error bars indicating the standard deviation.

binding while growth in the absence of histidine and the presence of 3AT (−His, +3AT) provides a more stringent test for binding. Growth in the presence of histidine (+His) is a control for cotransformation of the yeast with the expression constructs. WT Nef bound hemicomplexes of AP-1, -2, and -3 in a leucine-dependent manner (Fig. 7A), as previously described (4, 6, 22). Replacing the native Nef motif with that from the melanosomal protein tyrosinase, EERQPLL, yielded a Nef protein that, like the WT, bound to AP-1, -2, and -3. In contrast, substituting the motif from CD3γ, GDKQTLL, for the native Nef motif yielded a protein that bound only to AP-2.

To correlate these binding data with function, plasmids expressing these substitution-containing Nef proteins were transfected into HeLa-CIITA cells along with a plasmid expressing GFP as a transfection marker. Approximately 24 h later, the expression of Ii at the cell surface was assayed by flow cytometry. Cell surface Ii expression was increased in all cases in which a leucine-based motif was present in Nef (Fig. 7B). Nef-GDKQTLL (which bound only to AP-2) upregulated Ii with an activity equivalent to that of the WT. These data suggest that binding to AP-2 is sufficient for Nef to upregulate Ii and that AP-1 and AP-3 are unnecessary. Interestingly, Nef containing the leucine motif from the melanosomal protein tyrosinase (EERQPLL) increased the surface expression of Ii about threefold more than the WT, despite apparently similar binding specificities. These data lead to the hypothesis that

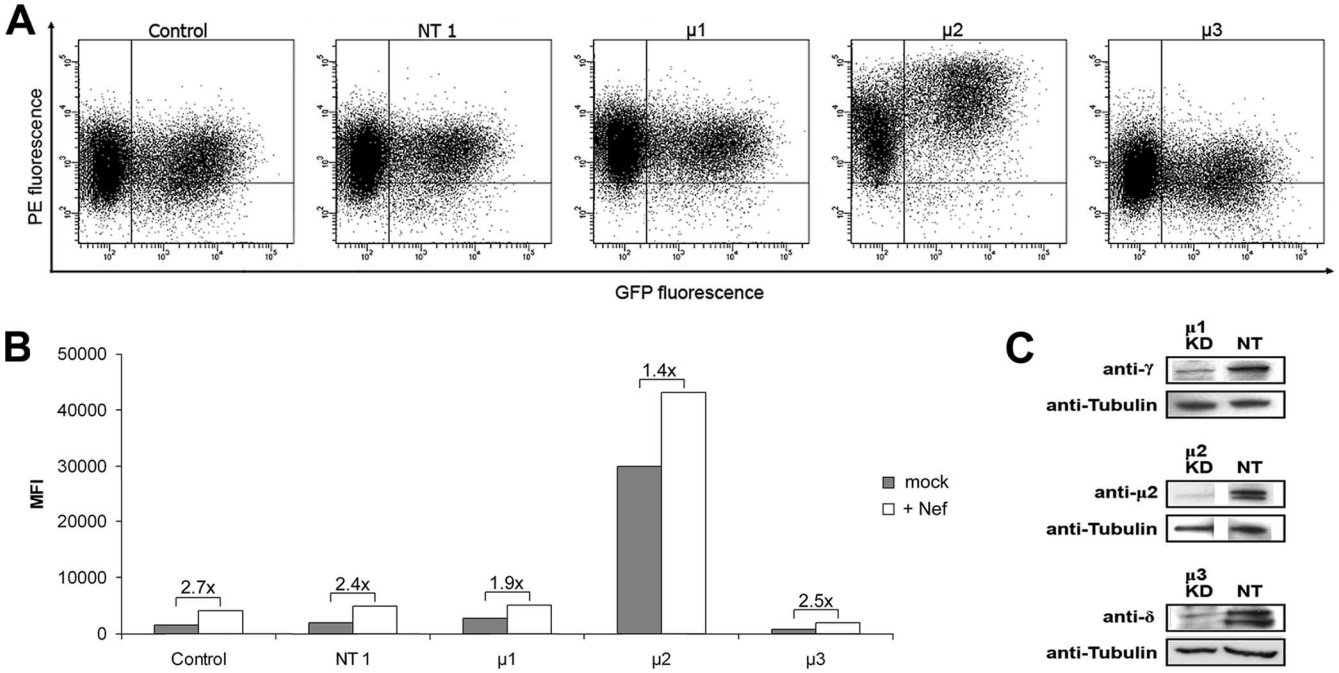


FIG. 8. Effects of knockdowns of AP complexes on Nef-mediated upregulation of cell surface Ii expression. HeLa-CIITA cells were transfected with siRNA duplexes that targeted the μ subunit of AP-1, AP-2, or AP-3. Three days later, the cells were cotransfected with siRNA duplexes and DNA plasmids that expressed GFP and Nef or the empty vector. Cells were harvested after 3 days, surface stained for Ii with a PE-conjugated Ab, and analyzed by flow cytometry. (A) Two-color fluorescence plots of cells transfected with DNA plasmids that express GFP; the empty vector (mock); siRNA targeting μ1, μ2, or μ3; or a nontargeting siRNA control (NT 1). Cells labeled "control" only received the DNA plasmids that express GFP and the empty vector. (B) PE MFI of high-expression $GFP^+$ cells from the two-color plots (GFP fluorescence of ≥1,200 on the *x* axis in panel A), as well as cells transfected as described above except that they received a Nef-expressing plasmid in place of the empty vector. The values above the brackets show the relative increases upon Nef expression. Data are representative of two independent experiments. (C) Immunoblot assays with anti-γ Ab for detection of AP-1, anti-μ2 Ab for detection of AP-2, and anti-δ Ab for detection of AP-3. KD, knockdown; NT, nontargeting control.

quantitative differences in the binding to specific complexes may account for the unusually high activity of this mutant Nef protein.

To test the hypothesis that Nef-EERQPLL binds to hemicomplexes of AP-2 with greater affinity than Nef-GDKQTLL and the WT, we studied these interactions by using quantitative Y3H assays. Cotransformed yeast cells were inoculated into selective liquid media, and the optical density at 600 nm was measured over time (Fig. 7C). Yeast expressing the Nef-EERQPLL mutant grew the most rapidly, followed by Nef-GENTSLL, Nef-GDKQTLL, and finally Nef-GENTSAA. These data indicated that Nef-EERQPLL has the greatest affinity for AP-2. Together with the relatively greater activity of Nef-EERQPLL in upregulating Ii, these data are consistent with the hypothesis that AP-2 is the key complex through which Nef modulates the trafficking of Ii.

**Suppression of AP-2 upregulates the expression of Ii at the cell surface and decreases the effect of Nef.** To further test the role of AP-2 in Ii trafficking and the upregulation of Ii by Nef, we used siRNAs to suppress the expression of specific complexes; RNA duplexes directed against μ1, μ2, and μ3 and a negative control were used to transfect HeLa-CIITA cells as described previously (4). A plasmid expressing GFP and either (i) a plasmid expressing Nef or (ii) an empty vector control were included in the second transfection along with the siRNA duplexes. Three days later, the cells were stained with an Ab to Ii and analyzed by flow cytometry. The use of siRNA targeting μ2 was associated with a striking 15-fold upregulation of Ii at the cell surface. In contrast, the use of siRNA targeting μ1 or μ3 had little or no effect (Fig. 8A) and immunoblots confirmed that the expression of the specific subunits of AP-1 (γ) and AP-3 (δ) was reduced (Fig. 8C). These data recapitulate the previously reported effects of siRNA inhibition of AP complexes on the expression of Ii at the cell surface (11, 33). Furthermore, when μ2 was targeted by siRNA, the upregulation of Ii at the cell surface by Nef was minimal (1.4-fold), whereas in the case of cells mock transfected or transfected with siRNA that targeted μ1, μ3, or an unrelated target (nontargeting control), the upregulation by Nef ranged from 1.9- to 2.7-fold (average of 2.1-fold; Fig. 8B). Although the effect of Nef was modest in this experimental format, it was reproducibly decreased only when μ2 was targeted, an intervention that itself upregulated the expression of Ii at the cell surface. An experiment in which flow cytometric analysis was performed 24 h after DNA transfection, the upregulation of Ii by Nef was 5-fold in cells transfected with the nontargeting siRNA and only 1.8-fold in cells transfected with the μ2-targeted siRNA (data not shown). These observations are consistent with an essential role for AP-2 in the trafficking of Ii and with a competition model in which Nef competes for AP-2 with Ii to induce upregulation.

## DISCUSSION

This study addressed how Nef increases the expression of Ii at the cell surface. We showed that the leucine-based AP-binding motif in Nef is not only necessary but also sufficient to upregulate Ii. Furthermore, most of the effect of Nef on Ii requires leucine-based sorting motifs in the cytoplasmic tail of Ii itself, without which Ii is expressed abundantly at the cell surface. RNAi knockdown of μ2 resulted in a 15-fold increase in the level of Ii at the cell surface, and the expression of Nef under these conditions led to a minimal further increase. The role of AP-2 was further supported by the observation that WT Nef decreased the fractional rate of internalization of Ii from the cell surface. These data are consistent with a model in which Nef competes with Ii for access to AP-2 complexes that would otherwise internalize and direct Ii to the MIIC. The data are also consistent with the observation that the cellular sorting machinery that recognizes leucine-based motifs is saturable (32). These mechanistic experiments were performed with HeLa-CIITA cells, but the Nef-mediated increase in Ii expression at the cell surface was also demonstrated in primary human $CD4^+$ T cells that were infected with WT HIV.

How does Nef downregulate some proteins while upregulating others, such as Ii? Models for the downregulation of cell surface proteins posit that Nef acts as a connector, linking the cytoplasmic tail of CD4 to AP-2 (4, 8, 14, 15, 29, 31) or the cytoplasmic domain of MHC-I to AP-1 (14, 29, 37, 48, 49). These proposed mechanisms require Nef to interact directly with CD4 or MHC-I and either an AP complex or another component of the cellular protein sorting machinery. Evidence for a direct interaction between Nef and a peptide sequence from the cytoplasmic tail of CD4 has been provided by nuclear magnetic resonance analysis (16), and a direct interaction between Nef and MHC-I has been supported by coimmunoprecipitation (49).

In contrast, for the Nef-mediated upregulation of Ii (and DC-SIGN, LIGHT, and tumor necrosis factor alpha [TNF-α]) (28, 44), we favor a model of competition between Nef and the membrane protein for binding to an AP complex. In this model, binding between Nef and the upregulated membrane protein is unnecessary. Indeed, no direct binding has been reported (28, 44, 47), perhaps because the presence of such binding is inconsistent with upregulation; if Nef bound these proteins, then they would presumably be directed to an endosomal compartment. Taking the connector and competition models together, two factors should predict if and how a cellular protein is affected by Nef, i.e., (i) whether or not the protein binds Nef and (ii) whether or not the protein has a constitutively active leucine-based AP-binding sequence. If the protein neither binds Nef nor has such a sequence, then it will be unaffected. If the protein binds Nef and does not contain a constitutively active leucine-based AP-binding sequence, then it will be downregulated from the cell surface; CD4 and MHC-I are prototypic examples. If the protein does not bind Nef but has a constitutively active leucine-based AP-binding sequence, then Nef will compete with it for binding to the AP complexes and the protein will accumulate on the PM by default. Ii, DC-SIGN, and membrane-bound TNF and LIGHT fall into this latter category. Additional support for this competition model is found in our previous work in which Nef was able to upregulate the cell surface expression of the Tac chimeras Tac-DKQTLL and Tac-ENTSLL (7).

Several features are common to the Nef-mediated upregulation of Ii, DC-SIGN, TNF, and LIGHT. Leucine-based sequences are present in the cytoplasmic tail of each of these proteins. Furthermore, Nef slows the internalization rate of DC-SIGN (44) and LIGHT (28) from the cell surface. We show here that Nef also slows the rate of internalization of Ii from the cell surface. Thus, the cause of increased cell surface expression of at least three of the four cellular membrane proteins currently known to be upregulated by Nef appears to be, at least in part, inhibition of internalization. Interestingly, we also note that Ii, DC-SIGN, TNF, and LIGHT are all type II membrane proteins whereas CD4 and MHC-I α chain are type I. Whether or how the orientation of these proteins in the membrane affects their responsiveness to Nef is unknown.

Experimental results herein support the model of competition between Nef and Ii for AP-2. Nef decreased the rate of internalization of Ii, and AP-2 is the AP complex associated with clathrin-dependent endocytosis. The mutant Nef protein containing the leucine-based motif from the CD3γ chain (GDKQTLL) bound only to the α/σ2 hemicomplex of AP-2 in Y3H assays, yet it was able to upregulate Ii as efficiently as WT Nef. Similarly, the Tac chimera containing the GDKQTLL sequence upregulated Ii at least as well as did the Tac chimera containing the native Nef sequence, GENTSLL. Knockdown of AP-2 increased the expression of Ii at the cell surface and decreased the apparent effect of Nef. The hypothesis that competition for and inhibition of AP-2-based trafficking of Ii is sufficient for upregulation is also consistent with the recent finding that AP-2 is the primary complex required for Ii to reach the MIIC (11, 33).

The mutant Nef protein containing the leucine-based motif from the melanosomal protein tyrosinase (EERQPLL), like WT Nef, bound to hemicomplexes of AP-1, AP-2, and AP-3 in the Y3H assay, yet this mutant protein upregulated Ii more efficiently than WT Nef did. Quantitative Y3H data suggested that this mutant Nef protein binds AP-2 with greater affinity than the WT does. Thus, stronger binding to AP-2 may explain why the Nef-EERQPLL mutant protein increases cell surface Ii expression more efficiently than WT Nef does. The conclusion that the EERQPLL sequence binds AP-2 with greater affinity than the GENTSLL sequence has recently been supported genetically: the binding of WT Nef to AP-2 requires a diacidic motif ($DD_{174,175}$) in the Nef C-terminal loop, yet this sequence is dispensable for the binding of a Nef-ERQPLL mutant to AP-2 (30). Notably, WT Nef and Nef-GDKQTLL show equivalent Ii upregulation activities yet WT Nef appears to bind AP-2 with slightly greater affinity than Nef-GDKQTLL does. These differences, though small, suggest that other factors besides affinity for AP-2 may contribute to the Nef-mediated upregulation of Ii.

Correlations between AP-binding and Ii upregulation activities drawn from the experiments herein further suggest that binding to AP-3 is unnecessary for the upregulation of Ii by Nef. This conclusion is consistent with studies indicating that the trafficking of MHC-II in mouse and human cells with genetic defects in AP-3 appears to be normal (3, 43). Furthermore, in the context of the competition model, the finding that a mutant Nef protein unable to bind AP-3 (Nef-GDKQTLL)

can still upregulate Ii is consistent with the previous report that the cytoplasmic tail of Ii does not bind AP-3 (21). Although up-regulation of Ii by Nef can apparently occur via competition for AP-2, Ii binds to AP-1 in vitro (21) and we cannot rule out the possibility that AP-1 also plays a role.

Previous mutational data indicate that the determinants of Ii upregulation in Nef are restricted to sequences in the C-terminal loop and include the $LL_{164,165}$ sequence, as well as the diacidic sequence $DD_{174,175}$ (42, 47). Although the DD sequence has been correlated with the binding of Nef to the H subunit of a vacuolar ATPase (13), recent data indicate that it is required for the direct binding of Nef to AP-2 but not to AP-1 or AP-3 (30). Together, these results also suggest that the interaction of native Nef with AP-2 is necessary to upregulate the Ii chain.

The data herein confirm the roles of specific residues in the Nef ExxxLL motif in the modulation of Ii and demonstrate that these roles are apparent either when Ii is expressed in isolation or when it is expressed in the context of the complete MHC-II. As in the cases of CD4, DC-SIGN, TNF, and LIGHT, modulation of Ii requires the leucine residues in the Nef motif. The acidic residue at position −4 relative to the leucines has an important role in the upregulation of Ii, consistent with its role in the efficient binding of Nef to AP complexes (4, 5). The data herein also confirm the key role of each of the leucine-based motifs in the cytoplasmic tail of Ii in Ii trafficking, and they further show that these sequences are crucial to Nef responsiveness. These observations are consistent with our central model in which the similar leucine-based motifs in Ii and Nef compete for AP-2. Interestingly, the mutant Ii protein in which both leucine-based motifs are disrupted ($L_7AL_{17}A$) was still upregulated by Nef, although only twofold. The mechanism of this effect is open to speculation, but this observation raises the possibility that competition between the leucine-based motifs of Ii and Nef for the cellular sorting machinery may explain most, but not all, of the upregulation. Alternatively, these single leucine mutant signals may have residual activity in binding to AP-2, although in vitro surface plasmon resonance studies have detected no binding between Ii-$L_7AL_{17}A$ and AP-2 (27).

In summary, this study provides evidence supporting a model in which Ii and Nef compete for binding to AP-2. In this model, Nef outcompetes Ii that has reached the cell surface for binding to AP-2, resulting in a decreased rate of endocytosis and an increase in the amount of Ii at the PM. The consequences of the Nef-mediated upregulation of Ii and the immature MHC-II complex in T cells and antigen-presenting cells for HIV pathogenesis remain to be elucidated.

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